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An Activated Form of Notch Influences the Choice between CD4 and CD8 T Cell Lineages

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Summary

Notch is a transmembrane receptor that controls cell fate decisions in Drosophila and whose role in mammalian cell fate decisions is beginning to be explored. We are investigating the role of Notch in a well-studied mammalian cell fate decision: the choice between the CD8 and CD4 T cell lineages. Here we report that expression of an activated form of Notch1 in developing T cells of the mouse leads to both an increase in CD8 lineage T cells and a decrease in CD4 lineage T cells. Expression of activated Notch permits the development of mature CD8 lineage thymocytes even in the absence of class I major histocompatability complex (MHC) proteins, ligands that are normally required for the development of these cells. However, activated Notch is not sufficient to promote CD8 cell development when both class I and class II MHC are absent. These results implicate Notch as a participant in the CD4 versus CD8 lineage decision.

Introduction

Cell–cell interactions play a crucial role in specifying cell fate during development. In the Drosophila embryo, cells are specified to become either neuronal or epidermal through interactions between neighboring cells. The transmembrane protein Notch is the receptor for a signal that diverts cells from adopting a primary, neuronal fate and directs them toward a secondary, epidermal fate instead. In Drosophila embryos that lack Notch function, all precursor cells become neuronal, whereas in embryos with a gain-of-function Notch mutation, extra epidermal cells appear at the expense of neurons (reviewed in Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995; Simpson, 1995). Although the role of Notch has been most extensively studied in the nervous system, Notch is expressed throughout Drosophila development and is involved in cell fate decisions in many different tissues.

Notch homologs have been identified in vertebrates, and the high degree of similarity in amino-acid sequence suggests a conserved role for Notch in vertebrate development (Coffman et al., 1990; Weinmaster et al., 1991; del Amo et al., 1992; Stifani et al., 1992; Weinmaster et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994). Consistent with this possibility, alterations in Notch function in vertebrates lead to developmental abnormalities (Jhappan et al., 1992; Coffman et al., 1993; Swiatek et al., 1994; Austin et al., 1995; Conlon et al., 1995) and cancer (Ellisen et al., 1991; Robbins et al., 1992; Pear et al., 1996). From these studies, however, there is no clear indication whether Notch affects the choice between alternative cell fates, as it does in invertebrates. In particular, while increased Notch activity reduces the number of cells that adopt a neuronal fate (Coffman et al., 1993; Austin et al., 1995), it is not clear whether the cells that received a Notch signal adopted alternative fates or simply failed to mature. In addition, these experiments do not provide any information about how Notch signals integrate with other developmental cues. Thus the issue of how Notch functions in vertebrates is still poorly understood.

We have begun to investigate the role of Notch in a well-studied mammalian cell fate decision: the choice between the CD4 and CD8 T cell lineages. In the thymus, developing T cells rearrange and express their T cell antigen receptor (TCR) genes and undergo a testing process based on the ability of their TCRs to recognize major histocompatability complex (MHC) proteins expressed on thymic epithelial cells (reviewed in Robey and Fowlkes, 1994; Jameson et al., 1995). The interaction between developing thymocytes and thymic epithelial cells promotes the survival of thymocytes and also directs their lineage choice. Thymocytes whose TCRs recognize class I MHC expressed on thymic epithelial cells develop as CD8 cells, whereas thymocytes whose antigen receptors recognize class II MHC proteins develop as CD4 cells. While it is clear that MHC recognition influences the choice between the CD4 and CD8 lineage, the mechanistic basis for this influence is not known.

The Notch1 gene is expressed in developing thymocytes (Weinmaster et al., 1991, 1992; Hasserjian et al., 1996), raising the possibility that Notch might also play a role in the CD4 versus CD8 lineage decision. We decided to investigate this possibility by examining the effect of a constitutively activated form of Notch on thymic development in the mouse. We find that expression of an activated form of Notch during thymic development biases the CD4/CD8 lineage decision in such a way that the development of CD8 T cells is favored over the development of CD4 T cells. In addition, activated Notch permits the development of CD8 lineage cells in the absence of class I MHC, a ligand that is normally required for CD8 cell development. However, activated Notch is not sufficient to promote CD8 cell development when both class I and class II MHC are absent. These results indicate that the Notch signaling pathway participates in the CD4 versus CD8 lineage decision. In addition, these results imply two distinct roles for MHC recognition in the development of CD4 and CD8 T cells: one that renders a thymocyte competent to respond to the Notch signal, and a second that acts together with Notch to direct the CD4 versus CD8 lineage decision.

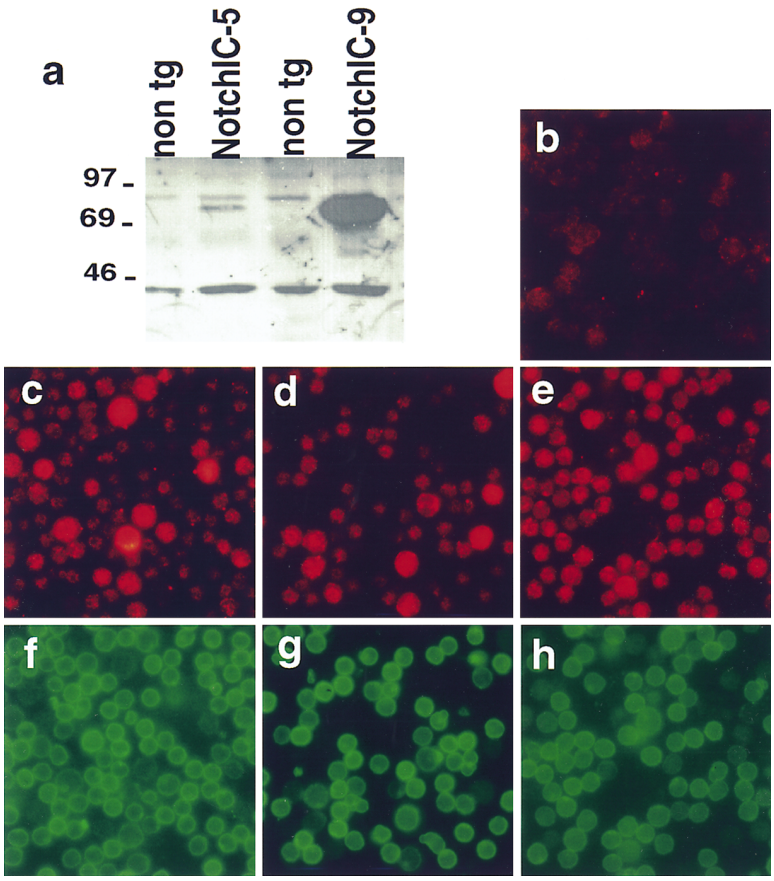

Figure 1. Expression of an Activated Notch Transgene and Endogenous Notch1 in Thymocytes

(a) Expression of the activated form of Notch (NotchIC) in thymocytes from transgenic mice. The transgenic construct used in this study consists of a portion of the Notch intracellular domain extending from amino acid 1750 to 2293, fused to an N terminal FLAG antibody epitope tag, under the control of the murine Lck proximal promoter (Chaffin et al., 1990; Abraham et al., 1991). This promoter directs high level, thymocyte-specific expression of heterologous coding regions in transgenic mice (Sentman et al., 1991; Teh et al., 1991). Immunoblot analysis using an anti-FLAG antibody of whole cell lysates (10^7 cell equivalents/lane) from thymocytes obtained from two transgenic mouse lines (NotchIC-5 and NotchIC-9) and their nontransgenic littermates. The positions of molecular-weight markers are indicated. A band corresponding to the approximate size of activated Notch is present in extracts from transgenic mice but not in extracts from nontransgenic mice. (b–h) Expression of endogenous Notch1 in thymocytes from wild-type mice. The Notch1 antisera was raised against a portion of the extracellular domain of Notch (amino acids 381–853) that is not included in the NotchIC transgene. (c and f) Class II MHC mutant mice (d and g) and NotchIC-9 transgenic mice (e and h) were doubly stained for expression of endogenous Notch1 (c–e) and CD4 (f–h). Control staining of wild-type thymocytes with an anti-GST antisera is also shown (b). Vertical panels depict the same field of cells.

Results

Transgenic Mice Expressing an Activated Form of Notch in Developing T Cells

Previous studies in invertebrates have established that truncated forms of Notch consisting of its isolated intracellular domain act as constitutively activated versions of the receptor and produce gain-of-function phenotypes when expressed during development (Lieber et al., 1993; Rebay et al., 1993; Roehl and Kimble, 1993; Struhl et al., 1993; Diaz-Benjumea and Cohen, 1995). For example, expression of the intracellular domain of Notch during Drosophila development under the control of a heat shock promoter leads to the development of extra epidermal cells at the expense of neurons in both the embryonic central nervous system and the adult peripheral nervous systems. These are the opposite phenotypes of those observed with loss-of-function mutations of Notch (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). Expression of the intracellular domain of Notch during wing development in Drosophila leads to the induction of wingless expression and the production of an ectopic wing margin, whereas loss-of-function mutations of Notch result in the loss of wingless expression and reduced growth at the wing margin (Diaz-Benjumea and Cohen, 1995). Similar observations have been made in C. elegans using truncated versions of the related receptors, lin-12 and glp-1 (Roehl and Kimble, 1993; Struhl et al., 1993). In these experiments, expression of the intracellular domain of either lin-12 or glp-1 leads to distinct cell fate transformations that are opposite to those observed with loss-of-function mutations. To investigate the role of Notch in T cell fate decisions, we therefore generated transgenic mice expressing the analogous activated form of Notch in developing thymocytes.

In order to express activated Notch in developing thymocytes, we first isolated the Notch1 cDNA from a mouse thymocyte cDNA library using a probe from the rat Notch1 gene (Weinmaster et al., 1991). We then generated a transgenic construct containing the intracellular domain of the murine Notch1 gene fused to a FLAG epitope tag, under the control of the thymocyte-specific Lck proximal promoter (Chaffin et al., 1990), and used this construct to generate transgenic mice. Anti-FLAG Western blot analysis of thymocyte extracts from two independent transgenic lines reveals the presence of a band corresponding to the approximate predicted size of activated Notch (Figure 1a).

Expression of Endogenous Notch in Thymocytes from Wild-Type Mice and from Mice Expressing an Activated Form of Notch

Previous studies indicate that Notch1 mRNA is expressed in the developing thymus (Weinmaster et al.,

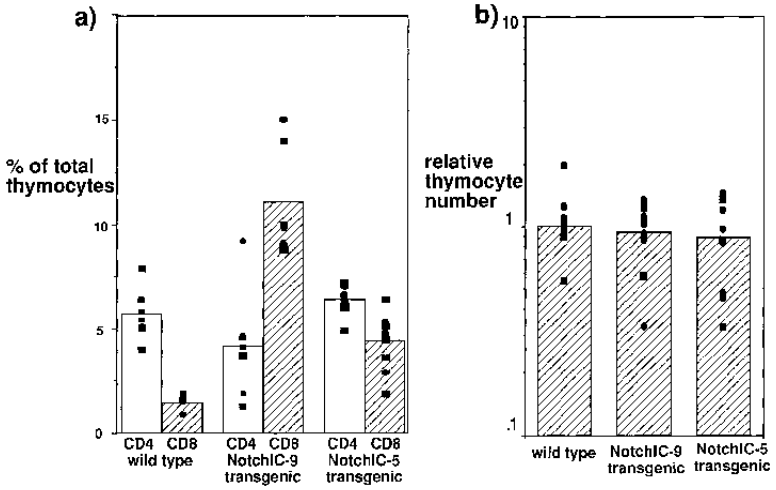

Figure 2. More CD8 Lineage Thymocytes in Mice Expressing an Activated Form of Notch

(a) The percentage of mature CD4 and CD8 lineage thymocytes in two lines of mice bearing the activated Notch transgene. Thymocytes from 4- to 12-week-old transgenic mice or nontransgenic littermates were analyzed for expression of CD4, CD8, and TCR. The gating of three parameter flow cytometric data was performed as shown in Figure 4. Mature CD4 lineage thymocytes are $CD4^+CD8^-TCR^{high}$, and mature CD8 lineage thymocytes are $CD4^-CD8^+TCR^{high}$. Bars are the average of the percentage in each population, and superimposed dots are values from individual mice. (b) Relative number of thymocytes in NotchIC transgenic mice. The total number of thymocytes from each mouse is normalized to the average of the nontransgenic littermates analyzed on the same day.

1991, 1992; Hasserjian et al., 1996). To confirm the presence of Notch1 protein in thymocytes and to examine the effect of activated Notch on endogenous Notch1 expression, we examined thymocytes from nontransgenic and NotchIC transgenic mice by immunofloures-cence, using antisera raised against a portion of the extracellular domain of Notch1. Thymocytes from wild-type mice have a fraction of cells that stain brightly with anti-Notch1 antisera (Figure 1c), a subset of which also expresses CD4 (Figure 1f). Mice that are deficient for class II MHC proteins also have a subset of thymocytes that express both Notch1 and CD4 (Figures 1d and 1g). Because class II MHC mutant mice lack mature CD4 lineage thymocytes, this indicates that some of the cells that stain brightly with the anti-Notch1 antisera are $CD4^+CD8^+$. In addition to the brightly staining cells, the majority of thymocytes from B6 and class II MHC mutant mice display a lower level of staining with the anti-Notch1 antisera that is significantly higher than the background observed with a control anti-GST rabbit antisera (Figures 1b–1d). This is consistent with previous flow cytometric analysis of Notch1 in thymocytes indicating that $CD4^-CD8^-$ cells stain brightly for Notch1, whereas $CD4^+CD8^+$ thymocytes display lower but signficant expression of Notch1 (Hasserjian et al., 1996). Interestingly, the staining on the majority of thymocytes is elevated in NotchIC transgenic mice (Figure 1e). Because the antibody is directed against a portion of the extracellular domain of Notch1 and the transgene consists of a portion of the intracellular domain, this increased staining reflects an increase in endogenous Notch1 protein in response to activated Notch. This suggests that Notch1 protein levels may be regulated by Notch signaling in thymocytes, a phenomenon that has been previously described for the Notch-like receptor lin-12 in C. elegans (Wilkinson et al., 1994).

Activated Notch Leads to an Increased Number of Mature CD8 Lineage Thymocytes

During thymic development, uncommitted T cell precursors give rise to the two major mature T cell lineages, CD4 T cells and CD8 T cells. To determine whether activated Notch affects this cell fate decision, we examined thymocytes from Notch transgenic mice for expression of CD4, CD8, and TCR. In normal mice, the majority of thymocytes are immature cells that express both CD4 and CD8 coreceptors and low levels of TCR. Mature CD4 and CD8 lineage thymocytes can be identified by expression of high levels of TCR and exclusive expression of either CD4 or CD8. We find that thymocytes from mice expressing the activated Notch transgene (NotchIC transgenic) have a 10-fold increase in the percentage of mature CD8 lineage thymocytes compared to their nontransgenic littermates (Figure 2a). Expression of the activated Notch transgene does not cause any significant alteration in the total number of thymocytes, most of which are immature $CD4^+CD8^+$ cells (Figure 2b). Thus there is an ~10-fold increase in both the absolute number and the percentage of mature CD8 lineage thymocytes in NotchIC-9 transgenic mice. The severity of the phenotype observed correlates with the level of expression of the activated Notch protein. The NotchIC-5 line, which expresses lower levels of activated Notch than the NotchIC-9 line (Figure 1a), shows a more modest increase (2.6-fold) in CD8 cells (Figure 2a). These results indicate that activated Notch favors the development of CD8 lineage T cells.

Reciprocal Changes in the Production Rate of CD4 and CD8 T Cells in Mice Expressing Activated Notch

In invertebrates, Notch and the related receptors lin-12 and glp-1 often control the choice between alternative cell fates. Since CD4 and CD8 lineage cells derive from a common thymic precursor, it was surprising to see an increase in CD8 lineage thymocytes in NotchIC transgenic mice without a consistent decrease in CD4 lineage cells. However, thymic development in the adult mouse is a dynamic process, and the steady-state size of the thymic populations is determined by several factors, including the rate at which cells are generated, the rate at which they die, and the rate at which they emigrate from the thymus. The steady-state size of the mature CD4 and CD8 populations in the thymus, therefore,

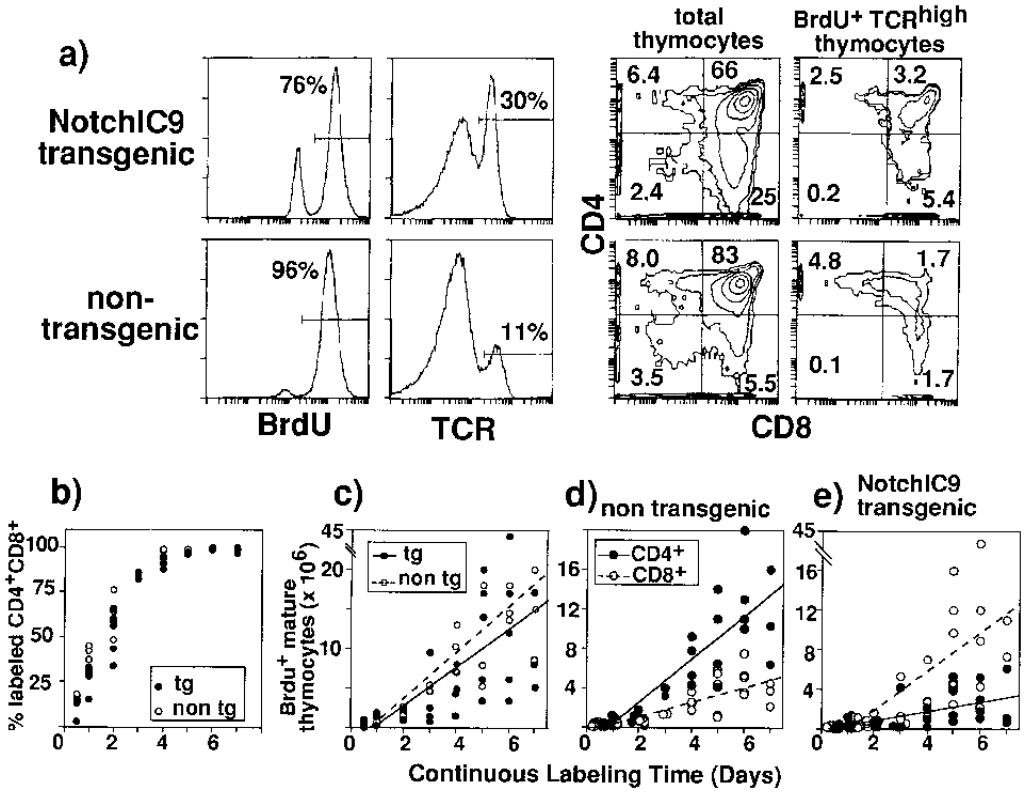

Figure 3. Kinetics of Appearance of Mature CD4 and CD8 Lineage Thymocytes in Mice Expressing the Activated Notch Transgene

Mice were continuously exposed to the thymidine analog bromodeoxyuridine (BrdU) in their drinking water for the indicated times. Thymocytes were then analyzed for BrdU content and for the expression of CD4, CD8, and αβTCR using four parameter flow cytometry, as described in Experimental Procedures. (a) Representative FACS plots for nontransgenic and NotchIC9 transgenic mice after 7 days of continuous BrdU labeling. The gates used to define the cell populations are indicated. (b) Turnover of $CD4^+CD8^+$ thymocytes. The percentage of $CD4^+CD8^+$ that are labeled with BrdU is plotted as a function of labeling time. (c) Time course of the appearance of mature labeled thymocytes from NotchIC transgenic mice (closed circles) or nontransgenic mice (open circles). Mature thymocytes are defined as TCR^{high} and either $CD4^+CD8^-$ or $CD4^-CD8^+$. The absolute number of mature labeled thymocytes of each type is plotted against the time of continuous exposure to BrdU. The rate of mature thymocyte production (calculated from a least squares fit of the data) is 2.9×10^6 cells/day for wild-type (R value = 0.86) and 2.4×10^6 cells/day for NotchIC transgenic mice (R value = 0.59). (d and e) Time course of the appearance of TCR^{high}, $Brdu^+$ cells that are either $CD4^+CD8^-$ (closed circles) or $CD4^-CD8^+$ (open circles). The absolute number of mature labeled thymocytes of each type is plotted against the time of continuous exposure to BrdU. The slopes of the lines are: nontransgenic CD4 cells, 2.2×10^6 cells/day (R value = 0.86); nontransgenic CD8 cells 0.74×10^6 cells/day (R value = 0.80); NotchIC transgenic CD4 cells, 0.47×10^6 cells/day (R value = 0.58); NotchIC transgenic CD8 cells 2.0×10^6 cells/day (R value = 0.54).

provides only an indirect indication of how many cells are choosing a particular fate.

To obtain a more direct measure of the effect of activated Notch on the CD4/CD8 lineage choice, we measured the kinetics of the appearance of mature thymocytes in NotchIC transgenic mice, using the thymidine analog bromodeoxyuridine (BrdU). We exposed NotchIC-9 transgenic and nontransgenic littermates to BrdU in their drinking water for various time periods and then analyzed thymocytes for the presence of BrdU and for the expression of CD4, CD8, and TCR (Figure 3). BrdU is incorporated in the DNA of dividing cells and can be detected using a monoclonal antibody. In wild-type mice, $CD4^+CD8^+$ thymocytes are short-lived cells that are proliferating or are the immediate products of proliferating cells. Thus $CD4^+CD8^+$ thymocytes label rapidly with BrdU, and almost all are $BrdU^+$ after 3–4 days of continuous labeling (Figure 3b). Importantly, the turnover of $CD4^+CD8^+$ thymocytes is virtually identical in NotchIC transgenic mice and wild-type mice. This indicates that activated Notch does not affect the proliferation or life span of immature $CD4^+CD8^+$ thymocytes.

The majority of $CD4^+CD8^+$ thymocytes die in the thymus, but a small fraction develop into long-lived mature thymocytes ($TCR^{high}CD4^+CD8^-$ or $TCR^{high}CD4^-CD8^+$). In contrast to $CD4^+CD8^+$ thymocytes, the mature thymic populations are not dividing, and this is reflected in the 1–2-day lag in the appearance of BrdU in these populations (Figures 3c–3e and Egerton et al., 1990). Importantly, this lag in the labeling of mature thymocytes is observed in both wild-type and NotchIC transgenic mice. This indicates that activated Notch does not lead to the inappropriate proliferation of mature thymocytes.

Because mature thymocytes are not proliferating, the rate of appearance of BrdU labels in these populations reflects the fraction of $CD4^+CD8^+$ precursors that develop into mature thymocytes. The rate of appearance of labeled mature thymocytes ($BrdU^+TCR^{high}$ and either $CD4^+CD8^-$ or $CD4^-CD8^+$) is similar in wild-type and NotchIC transgenic mice (Figure 3c). This indicates that

activated Notch does not significantly alter the fraction of thymocytes that develop into long-lived mature T cells. In contrast, the relative proportion of cells that develop as mature CD4 or CD8 lineage cells is dramatically affected by activated Notch (Figures 3d and 3e). NotchIC transgenic mice have an ~3-fold increase in the rate of CD8 cell production as well as an ~5-fold decrease in the rate of CD4 cell production, leading to a 15-fold change in the ratio of CD4 versus CD8 cell production compared to wild-type. Thus expression of an activated form of Notch in thymocytes leads to both an increase in the proportion of precursor cells that give rise to CD8 lineage cells and a decrease in the proportion that give rise to CD4 lineage cells. Although mature CD4 lineage thymocytes are generated less efficiently in NotchIC transgenic mice compared to wild type, the CD4 cells can sometimes accumulate to steady-state levels that are comparable to those found in wild-type mice (Figures 2a and 3a). This may reflect a homeostatic mechanism that regulates the steady-state numbers of mature CD4 T cells in the thymus. Together, these data indicate that activated Notch biases the choice of $CD4^+CD8^+$ cells between the CD4 and CD8 lineages in such a way that CD8 cell development is favored and CD4 cell development is inhibited. The involvement of Notch in a binary cell fate decision in the mammalian thymus is analogous to its well-documented role in the development of Drosophila and C. elegans.

Activated Notch Permits CD8 T Cell Development in Class I MHC Deficient Mice

The effect of the NotchIC transgene on the CD4/CD8 lineage decision implies that Notch activity favors CD8 T cell development over CD4 T cell development. However, it is well known that the choice between the CD4 and CD8 lineage is also controlled by binding of the TCR and the CD4 and CD8 coreceptors on developing thymocytes to MHC proteins on thymic epithelial cells (reviewed in Robey and Fowlkes, 1994; Jameson et al., 1995). Recognition of class I MHC by CD8 and TCR is required for the development of CD8 lineage cells, whereas recognition of class II MHC by CD4 and TCR is required for CD4 cell development. This is most clearly demonstrated by targeted gene-disruption experiments. For example, mice that are deficient for class I MHC expression due to a targeted disruption of the β2-microglobulin locus lack CD8 lineage T cells (Koller et al., 1990; Zijlstra et al., 1990). Because activated Notch favors CD8 lineage development, it is possible that activated Notch might override the normal requirement for class I MHC and allow CD8 lineage cells to develop in class I MHC deficient mice.

To investigate this question, we crossed the NotchIC transgenic mice with β2-microglobulin mutant mice and analyzed thymocyte populations from the littermates of the cross for expression of CD4, CD8, and TCR (Figures 4a–4e). In this experiment, we also examined the expression of the heat-stable antigen (HSA), a marker that is high on immature thymocytes and low on mature thymocytes. Mice that are heterozygous for the β2-microglobulin mutation, like wild-type mice, have a population of mature CD8 lineage thymocytes that are $CD4^-$, $CD8^+$, TCR^{high}, and HSA^{low} (Figure 4a). Mice that express the NotchIC transgene have ~10-fold more $CD4^-CD8^+$ thymocytes (Figures 2a, 4b, and 4e), and the majority of these cells express high levels of TCR and low levels of HSA (Figure 4b). In homozygous β2-microglobulin mutant mice, which are deficient for class I MHC, there are some $CD4^-CD8^+$ thymocytes, but these cells express low levels of TCR and high levels of HSA, indicating that these are the immature precursors of $CD4^+CD8^+$ thymocytes (Figure 4c). Strikingly, in homozygous β2-microglobulin mutant NotchIC transgenic mice, there is a large population of $CD4^-CD8^+TCR^{high}HSA^{low}$ thymocytes (Figures 4d and 4e). The mature CD8 population in class I MHC deficient NotchIC transgenic mice is reduced 3-fold compared to the mature CD8 population found in NotchIC transgenic mice that express class I MHC, suggesting that class I MHC does contribute to the development of the mature CD8 cells found in NotchIC transgenic mice. Nevertheless, class I MHC deficient NotchIC transgenic mice have 2-fold more mature CD8 thymocytes than do wild-type mice and greater than 100-fold more than do nontransgenic mice that lack class I MHC. These results indicate that activated Notch can drive the development of CD8 lineage thymocytes, even in the absence of class I MHC proteins.

Mature T cells that arise in the thymus emigrate to the peripheral lymphoid organs. To determine whether the CD8 T cells that develop in class I MHC deficient NotchIC transgenic mice can accumulate in the periphery, we examined lymph-node cells for expression of TCR, CD4, and CD8 (Figure 4f). NotchIC transgenic mice have ~25% of the normal number of lymph-node T cells and no consistent alterations in the CD4/CD8 T cell ratios (Figure 4f and data not shown). The Lck proximal promoter is known to be expressed at low levels in peripheral T cells (Chaffin et al., 1990; Teh et al., 1991). The reduced frequency of T cells in the lymph node of NotchIC transgenic mice may therefore be due to residual expression of activated Notch in mature T cells that may interfere with their function in the periphery. In class I MHC deficient mice, $CD8^+$ T cells represent <0.1% of lymph-node cells, whereas in class I MHC deficient mice expressing activated Notch, $CD8^+$ T cells make up 2% of lymph-node cells. Thus the CD8 lineage T cells that develop in NotchIC transgenic class I MHC deficient mice can accumulate in the periphery, albeit with a lower efficiency than CD8 T cells from wild-type mice.

Activated Notch Is Not Sufficient to Permit CD8 Cell Development in the Absence of Both Class I and Class II MHC Proteins

Thymocytes whose TCRs recognize class II MHC normally develop as CD4 lineage T cells. Because class II MHC proteins are present in β2-microglobulin mutant mice, the CD8 cells observed in NotchIC transgenic β2-microglobulin mutant mice could be cells with class II specific TCRs that developed as CD8 cells rather than CD4 cells due to the presence of activated Notch. To investigate this question, we made chimeric mice using donor hemopoetic stem cells from NotchIC transgenic mice to reconstitute irradiated host mice that were class I MHC deficient (β2-microglobulin mutant), class II MHC

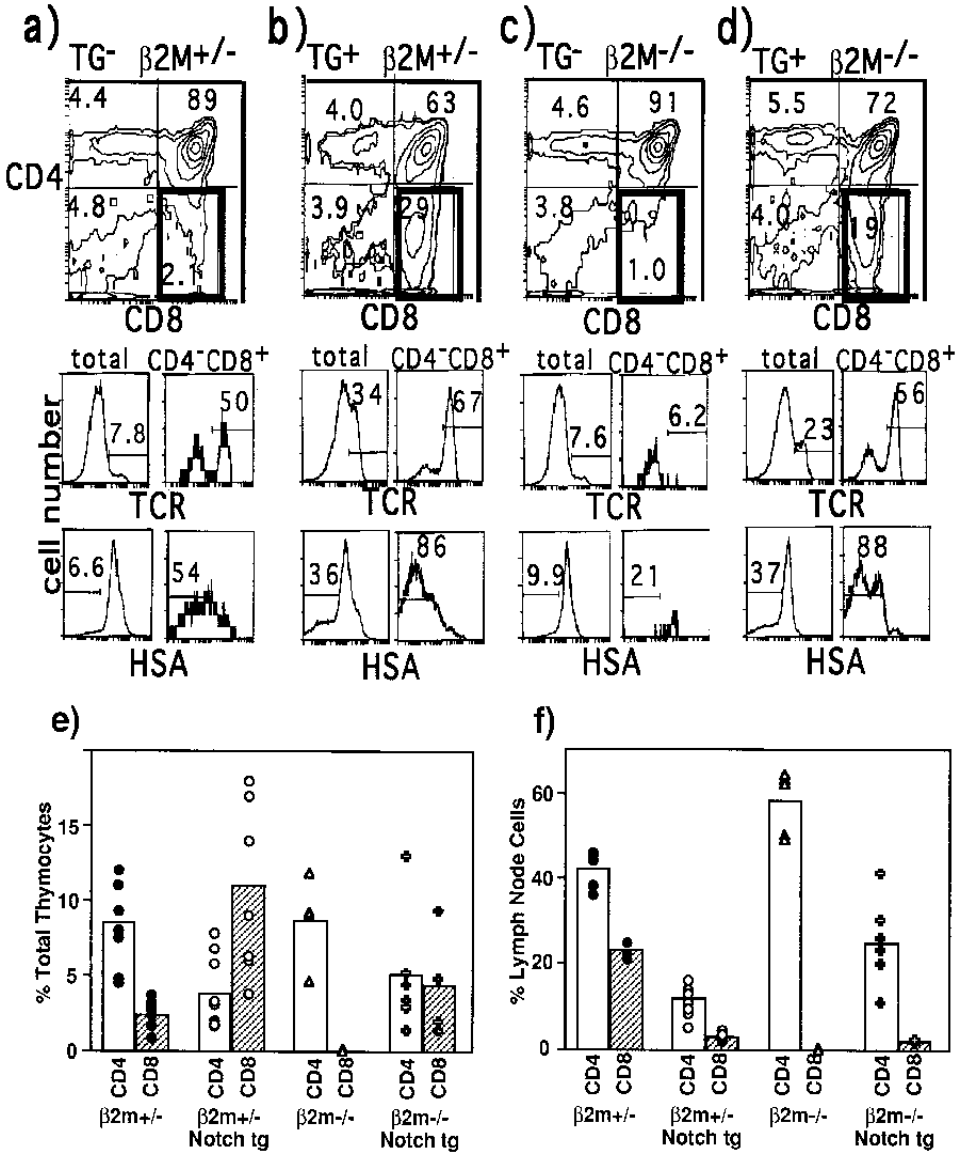

Figure 4. Activated Notch Permits the Appearance of Mature CD8 Lineage Thymocytes in the Absence of Class I MHC Proteins

Transgenic mice of the NotchIC-9 line were crossed with β2⁻microglobulin mutant mice (Zijlstra et al., 1990), and offspring from the second back-cross were typed for the presence of the NotchIC transgene (TG+) by dot blot hybridization and for homozygosity of the β2-microglobulin mutation (β2M⁻/⁻) by testing peripheral blood lymphocytes for expression of class I MHC proteins K^b and K^k. Thymocytes from 4- to 12-week-old mice from the second back-cross were analyzed for expression of CD4, CD8, TCR, and HSA. Representative FACS plots for (a) nontransgenic, β2-microglobulin +/−, (b) NotchIC-9 transgenic, β2-microglobulin +/−, (c) nontransgenic, β2-microglobulin −/−, and (d) NotchIC-9 transgenic, β2-microglobulin −/− are shown. The numbers in the quadrants indicate the percentage of total thymocytes in each population. The dark rectangle indicates the analysis gate used to define $CD4^-CD8^+$ thymocytes. The numbers over bar are the percentage of thymocytes within the indicated gates. (e) The percentage of thymocytes that are mature CD4 or CD8 lineage thymocytes. The genotypes of the mice are indicated. Mature CD4 lineage thymocytes are $CD4^+CD8^-TCR^{high}$, and mature CD8 lineage thymocytes are $CD4^-CD8^+TCR^{high}$. Bars are the average of the percentage in each population, and superimposed dots are values from individual mice. (f) The percentage of lymph-node cells that are mature $CD4^+CD8^-$ or $CD4^-CD8^+$. The genotypes of the mice are indicated.

deficient (Aβ mutant mice [Cosgrove et al., 1991; Grusby et al., 1991]), or class I and class II MHC deficient (β2-microglobulin, Aβ mutant mice). As previously observed, when wild-type thymocytes develop in class I MHC deficient hosts, very few mature CD8 cells develop (Figure 5a) (Bix and Raulet, 1992). This reflects the fact that CD8 T cell development is largely dependent upon the recognition of class I MHC proteins on the radiation-resistant thymic epithelial cells of the host. When wild-type T cells develop in class II MHC mutant hosts, very few mature CD4 cells develop (Figure 5b) (Markowitz et al., 1993), whereas in MHC double mutant hosts, few mature T cells of either lineage are present (Figure 5c). In contrast, a substantial population of mature CD8 thymocytes is present when NotchIC transgenic thymocytes develop in either class I MHC deficient (Figure 5d) or class II MHC deficient (Figure 5e) hosts (5.8% and 3.9% of total thymocytes, respectively). However, when NotchIC transgenic T cells develop in hosts that lack expression of both class I and class II MHC, very few

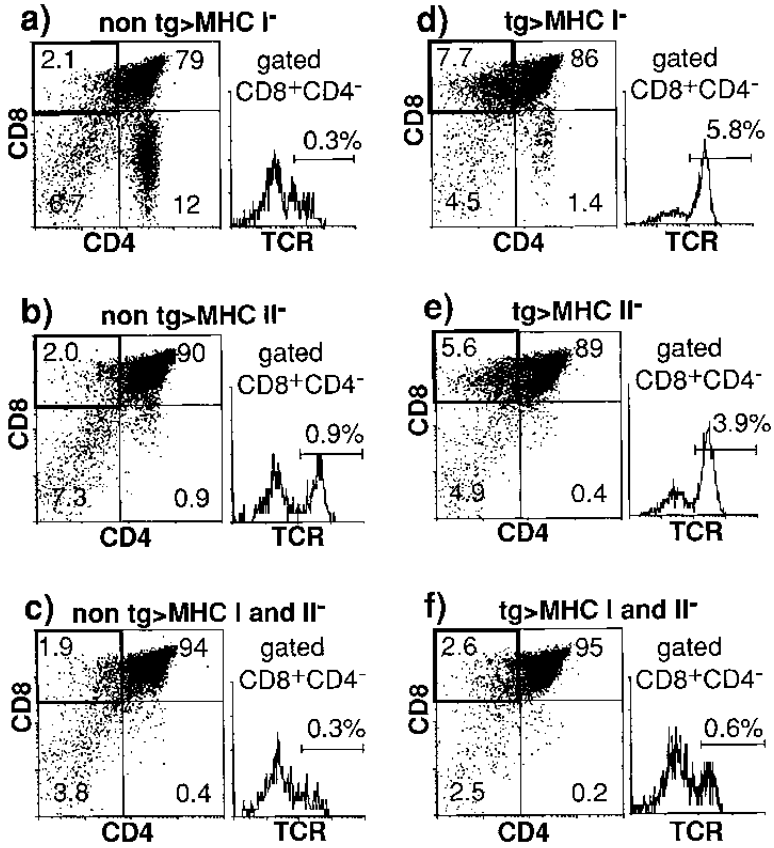

Figure 5. Either Class I or Class II MHC Is Required for the Development of CD8 Lineage T Cells in Thymocytes Expressing Activated Notch

Hemopoetic stem cells from nontransgenic (a–c) or NotchIC-9 transgenic (d–f) mice were used to reconstitute irradiated MHC mutant mice. Irradiated mutant hosts were class I deficient (a and d) (β2-microglobulin mutant mice, Zijlstra et al., 1990), class II deficient (b and e) (Aβ mutant mice, Grusby et al., 1991), or MHC class I and class II deficient (c and f). After 4 weeks to allow reconstitution of the thymus, thymocytes from radiation chimeras were analyzed for expression of CD4, CD8, and αβTCR, as described in Experimental Procedures. The numbers in the quadrants indicate the percentage of total thymocytes in each population. The dark rectangle indicates the analysis gate used to define $CD4^-CD8^+$ thymocytes. The numbers over bar are the percentage of total thymocytes. Two chimera of each type were analyzed, and representative data are shown. The small population of mature CD8 cells present in wt > class I MHC deficient chimeras could be due to the recognition of class I MHC proteins of the donor cells (Bix and Raulet, 1992).

mature CD8 thymocytes are observed (Figure 5f). Together, these results indicate that activated Notch alters the fate of the maturing T cells but does not overcome the requirement for MHC recognition. When Notch is regulated normally, class I recognition leads to CD8 cell development and class II MHC recognition leads to CD4 cell development. In contrast, in the presence of a constitutively activated form of Notch, recognition of either class I or class II MHC leads to the development of CD8 lineage T cells.

Discussion

The choice of uncommitted thymocytes between the CD4 and CD8 T cell lineages is governed by the recognition of MHC class I and class II proteins on thymic epithelial cells, but the mechanism that drives this lineage decision is unknown. Here we show that expression of an activated form of Notch in developing T cells biases the choice between the CD4 and CD8 lineages in such a way that CD8 cell development is favored over CD4 cell development. When Notch is regulated normally, the recognition of class I MHC by developing thymocytes leads to CD8 T cell development, whereas class II MHC recognition leads to CD4 T cell development. In the presence of activated Notch, however, recognition of either class I or class II MHC leads to CD8 T cell development. These results show that alterations in Notch activity can affect the choice between two alternative mature cell fates in mammals. Furthermore, these results have important implications for understanding how MHC recognition influences the CD4 versus CD8 lineage choice.

In invertebrates, the role of Notch in cell fate decisions has been well documented using conditional loss-of-function and gain-of-function mutations. It is important to note that the phenotypes observed when the intracellular domain of Notch is expressed during development correspond to the reciprocal cell fate transformations to those observed with loss of function alleles in every case described (Lieber et al., 1993; Rebay et al., 1993; Roehl and Kimble, 1993; Struhl et al., 1993; Diaz-Benjumea and Cohen, 1995). It is quite likely, therefore, that the phenotype that we observe reflects a normal physiological process mediated by Notch. Furthermore, the highly specific effect of the mutation on the CD4/CD8 cell fate decision but not on the proliferation, turnover rate, or steady-state number of thymocytes also strongly suggests a specific role for the Notch signaling pathway in CD4/CD8 cell fate determination. Although our experiments do not prove that Notch is involved in this decision, the fact that Notch1 is expressed in developing thymocytes (Weinmaster et al., 1991, 1992; Hasserjian et al., 1996; and Figure 1, here) makes this quite likely. Analysis of the effect of a Notch1 gene disruption on thymic development may provide a resolution of this question. Because Notch1 mutant mice die around day 10 of gestation, before the formation of the thymus (Swiatek et al., 1994; Conlon et al., 1995), a conditional gene disruption is needed to definitively assess the role of Notch1 in T cell development.

How do Notch and MHC recognition work together to specify the CD4 and CD8 T cell fates? While we do not yet have a complete answer, the effect of activated Notch in MHC mutant mice provides an important clue. The observation that an activated form of Notch can

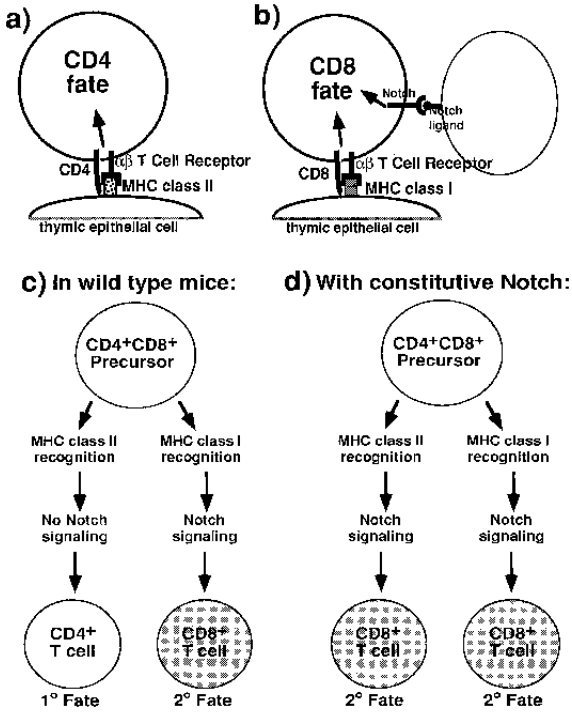

Figure 6. Signal Integration in the CD4 versus CD8 Lineage Decision

(a) A thymocyte that recognizes class II MHC avoids the Notch signal. (b) A thymocyte that recognizes class I MHC receives the Notch signal. We have depicted the ligand for Notch on a neighboring thymocyte (lateral signaling); however, an alternative possibility is that the ligand for Notch is on another cell type (inductive signaling) (Henderson, 1994). (See text for discussion.) (c) In wild-type mice, in which Notch is regulated by ligand binding, recognition of class II MHC allows the thymocyte to avoid the Notch signal, and thymocytes develop as CD4 lineage T cells. In contrast, thymocytes that recognize class I MHC proteins receive the Notch signal and develop as CD8 cells. (d) In the presence of a constitutively activated form of Notch, thymocytes that recognize class I or class II MHC receive a Notch signal and develop as CD8 cells.

cause mature CD8 T cells to develop in class I MHC deficient mice suggests that Notch acts downstream or in a parallel pathway with MHC recognition to specify the CD8 T cell fate (Figure 6). This implies that when a thymocyte recognizes a class I MHC, it results in the cell receiving a Notch signal. Conversely, when a thymocyte recognizes class II MHC, it allows the cell to avoid the Notch signal. In the presence of a constitutively activated form of Notch, thymocytes that recognize class II MHC can no longer avoid the Notch signal and develop as CD8 cells.

How might Notch signaling be influenced by MHC recognition? Notch is normally regulated by ligand binding, and a number of scenarios could be envisaged by which MHC recognition would determine whether or not a thymocyte is exposed to the Notch ligand. One possibility is that there are two different types of specialized thymic epithelial cells: those that express both class I MHC and the Notch ligand and those that express class II MHC but not the Notch ligand. Thus thymocytes that recognize class I MHC would come into contact with the ligand and those that recognize class II MHC would not. Another more interesting possibility is that both Notch and the Notch ligand are expressed on thymocytes, and Notch signaling between neighboring thymocytes is influenced by signals through the TCR and CD4 or CD8 coreceptors upon recognition of MHC proteins on thymic epithelial cells (Figures 6a and 6b). Recognition of class I and class II MHC generates distinct intracellular signals, due in part to the participation of CD8 and CD4 (Ravichandran and Burakoff, 1994; Itano et al., 1996). Perhaps signaling through CD4 causes a thymocyte to down-regulate Notch, whereas a CD8 signal makes a thymocyte up-regulate Notch. An initial difference in the level of Notch signaling due to class I or class II MHC recognition might then be reinforced by a feedback mechanism, analogous to those described in Drosophila (Heitzler and Simpson, 1991) and C. elegans (Wilkinson et al., 1994), wherein increased Notch signaling results in up-regulation of Notch and down-regulation of the Notch ligand. Indeed, the observation that endogenous Notch1 protein is increased in the presence of activated Notch is consistent with such a mechanism. This model is also reminiscent of vulva development in C. elegans, in which the choice of precursor cells between primary and secondary cell fates is determined by the combined action of a graded inductive signal (acting through a receptor tyrosine kinase) and a lateral signal through the Notch-like receptor lin-12 (Beitel et al., 1995; Katz et al., 1995). With the recent identification of ligands for Notch in vertebrates (Bettenhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Lindsell et al., 1995), soon the thymic ligand for Notch should be identified and the manner of regulating Notch signaling in the thymus resolved.

Although activated Notch can allow thymocytes bearing class II specific TCRs to develop as CD8 T cells, it cannot allow CD8 cell development in the complete absence of MHC recognition. This implies a role for MHC recognition that is distinct from its role in directing the CD4 versus CD8 lineage decision. The vast majority of developing T cells die in the thymus, due in part to the requirement for MHC recognition for thymocyte survival. In the absence of an MHC signal, a thymocyte may not survive long enough to differentiate into a CD8 cell, even though it may receive a Notch signal. It is also possible that MHC recognition induces a maturation program that can lead to the development of either CD4 or CD8 lineage cells. This program would then be influenced by the combined action of Notch and a specific signal through either class I or class II MHC recognition to direct the developing T cell to the appropriate lineage.

Experimental Procedures

The mouse Notch1 cDNA was isolated from a mouse thymocyte cDNA library using the rat Notch1 probe (kindly provided by Gerry Weinmaster; see Weinmaster et al., 1992). Partial sequence analysis of 16 hybridizing clones indicated that they all corresponded to mouse Notch1 (del Amo et al., 1993). A synthetic linker containing a Kozak sequence, AUG, and a FLAG epitope was inserted before the NgoMI to SpeI fragment of the mouse cDNA and cloned into the EcoRI and SpeI sites of Bluescript. The sequence of the sense oligo is: 5′-aa ttc ggt acc atg gac tac aaa gac gat gac gat aaa gc-3′. The anti-sense oligo is: 5′- ccg ggc ttt atc gtc atc gtc ttt gta gtc cat ggt acc g-3′. The 1.7 kb EcoRI to HindIII fragment of this plasmid

containing NotchIC coding regions and linker sequences was then blunt-end ligated into the BamHI site of a transgenic expression vector (p1017, kindly provided by Roger Perlmutter) consisting of the mouse Lck proximal promoter and human growth hormone coding sequences and polyadenylation site (Chaffin et al., 1990). The NotchIC fragment included in the transgenic construct contains the ankyrin repeat region and the nuclear localization sequences (Stifani et al., 1992) but does not include the C terminal PEST and *opa* sequences. An SpeI fragment containing the entire insert without vector sequences was purified and injected into (C57Bl/6 × CBA/J)F2 embryos. Transgenic founders were identified by Southern blot analysis and maintained by back-crossing to C57Bl/6. We occasionally observe thymomas in older NotchIC-9 transgenic mice; however, most transgenic mice remained healthy up to 12 weeks after birth (unpublished data). In mice with tumors, the number of thymocytes was increased and the majority of thymocytes displayed an abnormal cell surface phenotype (predominantly $CD8^+CD4^{low}$, TCR^{low}). All of the mice used in this study were 4–12 weeks of age, appeared healthy, and had normal thymocyte numbers and cell surface phenotype.

Whole cell lysates were made by resuspending 10^8 thymocytes from 6- to 8-week-old mice in 150 ml lysis buffer containing 50 mM Tris (pH 7.5), 10 mM NaF, 10 mM NaPyr, 200 mM NaVan, and 1% NP-40. Lysate from 10^7 cells was separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with a mouse anti-FLAG M2 antibody (10 μg/ml; VWR) and a goat anti-mouse IgG peroxidase conjugate secondary antibody (1/2000; Southern Biotech), according to the recommendations of the manufacturer, and detected using an ECL peroxidase substrate.

Cell suspensions of thymocytes were prepared and labeled with fluorescent antibodies as previously described (Itano et al., 1996). Antibodies used were PE-labeled CD4 (Beckton-Dickenson), FITC-labeled CD8α (YTS 169.4, Caltag), FITC-labeled anti-αβTCR (Pharmingen), biotinlyated anti-CD8 (Caltag), anti-Heat Stable Antigen (J11.d culture supernatant), R613-labeled anti-RatIg (GIBCO–BRL), Tricolor labeled-Streptavidin (Caltag), and rat gamma-globulin (CalBiochem, San Diego, CA). Data (50,000 events) were collected and analyzed using an X-cell flow cytometer (Coulter). Dead cells were excluded on the basis of forward and side scatter.

For immunoflourescence analysis, thymocytes were plated on poly-D-lysine (200 μg/ml)-coated coverslips and fixed using 4% paraformaldehyde at room temperature for 30 min. The coverslips were then blocked in 10% normal goat serum, 0.4% Triton X-100, and 3% bovine serum albumin for 30 min at room temperature. The coverslips were next incubated sequentially with the following antibodies: affinity-purfied anti-Notch1 antisera (1:150), biotinylated anti-rabbit Ig (Vector), Texas red-labeled avidin (Zymed), rat gamma-globulin, and FITC-labeled anti-CD4 (Pharmingen). The anti-Notch antisera was raised against a GST fusion protein containing amino acids 381–853 of rat Notch1. Details of the production and characterization of the Notch1 antisera will be reported elsewhere (C. Shawber and G. Weinmaster, unpublished data). Anti-GST antisera (kindly provided by Y. Zhang and J. Allison) was used at 1 μg/ml.

For kinetic analysis of thymic development, mice were continuously exposed to the thymidine analog bromodeoxyuridine (0.8 mg/ml) in their drinking water for various times. Extracellular labeling of thymocytes with antibodies against CD4, CD8, and αβTCR was performed as described above using R613-labeled anti-CD4 (GIBCO–BRL), biotinlyated anti-CD8 (Caltag), and PE-labeled anti-αβTCR (Caltag). Cells were then fixed and stained with FITC-labeled anti-BrdU (Beckton-Dickenson) as previously described (Tough and Sprent, 1994). The absolute number of labeled thymocytes in each population was determined by multiplying the percentage of cells with a particular phenotype times the total number of thymocytes. The rate of production of mature thymocytes was calculated from a least squares analysis of data points from 2–7 days of continuous labeling.

Bone marrow chimeras were constructed using T depleted bone marrow cells from NotchIC-9 transgenic or nontransgenic mice. MHC mutant mice were given 900 rads of γ irradiation (Cs source), and donor stem cells (10^7) were injected intravenously 4–18 h later. Radiation chimeras were maintained on antibiotic water and analyzed 4–5 weeks after reconstitution.

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